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# Further Development of Rodent Whole Embryo Culture: Solvent Toxicity and Water Insoluble Compound Delivery System

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16. ABSTRACT

In order to study the in vitro embryotoxicity and dysmorphogenesis of water insoluble compounds, solvents or chemical delivery systems of low toxicity and teratogenicity to the developing embryo must be found. Therefore, day 10.5 rat embryos were cultured for 2 days in whole rat serum containing 0.1, 0.5 and 2.5 vol.% of ethyl alcohol, dimethylsulfoxide, acetone, Tween 80, corn oil and 10% acetone/90% corn oil. No adverse effects occurred with any of the solvents at the 0.1% concentration level. At 0.5% ethyl alcohol and Tween 80 significantly reduced embryonic growth and increased the incidence of embryonic abnormalities. With the exception of corn oil and acetone/corn oil, embryos cultured in media containing 2.5% of various solvents failed to grow, did not differentiate, and died during the culture period. Corn oil suspended in rat serum by use of ultrasound was non-toxic even at concentrations of 2.5% and 10%. Growth parameters of embryos cultured in serum containing corn oil were indistinguishable from controls and overall morphogenesis was good (particularly at 2.5%). The order of increasing embryotoxicity and dysmorphogenesis of the studied liquids was corn oil < acetone/corn oil < dimethylsulfoxide < ethyl alcohol, acetone <

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# FURTHER DEVELOPMENT OF RODENT WHOLE EMBRYO CULTURE: SOLVENT TOXICITY AND WATER INSOLUBLE COMPOUND DELIVERY SYSTEM

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SUMMARY

In order to study the in vitro embryotoxicity and dysmorphogenesis of water insoluble compounds, solvents or chemical delivery systems of low toxicity and teratogenicity to the developing embryo must be found. Therefore, day 10.5 rat embryos were cultured for 2 days in whole rat serum containing 0.1, 0.5 and 2.5 vol.% of ethyl alcohol, dimethylsulfoxide, acetone, Tween 80, corn oil and 10% acetone/90% corn oil. No adverse effects occurred with any of the solvents at the 0.1% concentration level. At 0.5% ethyl alcohol and Tween 80 significantly reduced embryonic growth and increased the incidence of embryonic abnormalities. With the exception of corn oil and acetone/corn oil, embryos cultured in media containing 2.5% of various solvents failed to grow, did not differentiate, and died during the culture period. Corn oil suspended in rat serum by use of ultrasound was non-toxic even at concentrations of 2.5% and 10%. Growth parameters of embryos cultured in serum containing corn oil were indistinguishable from controls and overall morphogenesis was good (particularly at 2.5%). The order of increasing embryotoxicity and dysmorphogenesis of the studied liquids was corn oil < acetone/corn oil < dimethylsulfoxide < ethyl alcohol, acetone <

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*Abbreviation:* DMSO, dimethyl sulfoxide.

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Tween 80. Any of the 4 water miscible solvents (at 0.1%) or a sonicated suspension of corn oil in serum (up to 2.5%) met the criteria of a non-toxic and non-teratogenic water insoluble compound delivery system for in vitro embryo culture.

*Key words:* Embryo culture; Solvent toxicity; In vitro teratology; Ethyl alcohol; Dimethyl sulfoxide

## INTRODUCTION

Culture of rodent whole embryos was first achieved by Nicholas and Rudnick [1]; recent studies by New [2] and his co-workers [3] have greatly improved the methodology of this in vitro technique. In vitro whole embryo culture has obvious mechanistic uses in experimental teratology for solving problems of maternal-embryonic interactions. New [4] suggested that in vitro culture of whole embryos could be used as a teratogenic screening technique since the substantial growth and morphogenesis of rat embryos closely matches that of in vivo controls during the day 9.5–12.5 period of organogenesis. However, for rodent whole embryo culture to be a versatile, sensitive in vitro technique for detecting embryotoxins or teratogens, further methodological improvements are necessary.

In some cases the metabolites of compounds are more embryotoxic and teratogenic than the parent compound. Since embryonic tissues are generally considered to have little or no metabolic activation capacity (e.g., lack of a cytochrome *P*-450 system), the coupling of adult hepatic metabolic activation systems to whole embryo culture was accomplished [5–7]. Recently, Filler and Lew [8] have shown that preimplantation mouse embryos have a small but measurable ability to metabolize benzo[$\alpha$]pyrene. This metabolic activation system allows for the direct detection of proteratogens and protoxins like cyclophosphamide. An alternative indirect approach, uses in vivo activation of compounds to produce teratogenic activity in the serum of rats [9] or humans [10]. A third approach exposes rodent embryos to chemicals in vivo followed by an in vitro culture period [11].

A remaining problem in using whole embryo culture as a teratogenic screening system is the development of techniques to introduce water insoluble compounds into the aqueous culture media of developing conceptuses. Solvents such as dimethyl sulfoxide (DMSO) [12] and ethyl alcohol [13] are known teratogens in either animal or man. To date, few in vitro whole embryo culture studies have been published using water insoluble compounds. Galloway et al. [14] cultured early mouse embryos with up to 10.0 $\mu$M benzo[$\alpha$]pyrene dissolved in DMSO (up to 0.8%). This concentration of benzo[$\alpha$]pyrene is substantially below that usually employed to saturate in vitro hepatic microsomal activating systems (80 $\mu$M) [15] or to produce embryo toxicity or teratogenicity in vivo (about 200 $\mu$M/kg) [16]. With day 14.75 mouse embryos, Wee et al. [17] found 1.0% DMSO stimulated posterior palate shelf rotation, the biological system under investigation. The possible

interaction between toxic or teratogenic solvents (the chemical agents under study here) and their solutes must be considered. For example, the nature and amount of the solvent alters the in vivo teratogenicity of 3-acetylpyridine [18,19].

In this report, experiments were performed to determine the in vitro rat embryotoxicity and dysmorphogenesis of 4 common water miscible solvents: ethyl alcohol, DMSO, acetone and Tween 80 at 0.1%, 0.5% and 2.5%. In addition, the water insoluble solvent corn oil has been suspended in the embryo culture media in concentrations of 0.1%, 0.5%, 2.5% and 10% by use of ultra sound. As the combination of 10% acetone and 90% corn oil has been used successfully as a vehicle for water insoluble compounds in various in vivo animal experiments [20], this combination was also used in these in vitro studies. The embryotoxicity and teratogenicity of the 6 solvents are discussed in respect to: (i) the development of a water insoluble compound delivery system for in vitro embryo culture; and (ii) the available in vitro and in vivo literature.

## MATERIALS AND METHODS

### *Whole embryo culture*

Male and female Sprague–Dawley rats of the CD strain were housed together overnight, and the following morning vaginal smears were made. Females with sperm-positive smears were determined to be at day 0.5 of gestation. On day 10.5 post coitus pregnant rats were killed by guillotine and the abdominal cavity opened to expose the gravid uterus. Under aseptic conditions, conceptuses were removed from the uterus and collected in petri dishes containing Trowell's T8 media. The decidual tissue and Reichert's membrane were removed by the technique described by Sanyal and Wiebke [21]. Whenever possible, embryos harvested from the same dam were put in different treatment groups for concurrent culturing of control and solvent exposed embryos.

Blood was drawn from the dorsal aorta of 90-day-old male CD rats, immediately centrifuged, allowed to stand at room temperature for 1 h and then recentrifuged for 30 min. The serum was stored at −20°C until needed (usually within a 2-week period). Prior to its use, the serum was thawed, filtered and heat inactivated at 56°C for 30 min [2,3]. The serum was allowed to cool and 50 units/ml of penicillin and 50 $\mu$g/ml of streptomycin were added.

Each explant was individually cultured for 48 h in a 30-ml serum bottle containing 5.0 ml of culture medium and a mixture of gases (5.0% $CO_2$; 95% air on day 10.5, followed by 5.0% $CO_2$, 40.0% $O_2$; 55.0% $N_2$ on day 11). Each culture bottle was rotated on a roller culture assembly at 60 rev./min in a 37°C incubator. At the end of the culture period, explants were removed from the culture vessel and observed microscopically. The explants were evaluated for growth and differentiation using criteria which included increased yolk sac diameter and circulation, crown-rump length, head length and number of somites. Some of the embryos and yolk sacs were stored at −20°C for later

quantification of DNA [22] and total protein using a semi-automated Lowry procedure [23] in a Union Carbide Centrifichem 400 system. Embryos without a beating heart or functional yolk sac circulation were classified as dead. All experimental results were discarded if the viability of control embryos fell below 65% on a given experimental day. In the assessment of morphology, the embryonic flexion, heart, neural tube, brain development, otic, optic and olfactory systems, branchial bars, mandibular and maxillary processes, and fore and hind limb buds were examined. Morphogenesis was scored normal or abnormal as compared to untreated controls. Embryos grown in undiluted serum or serum containing up to 10% $H_2O$ grew and differentiated similarly.

*Chemicals and statistical analysis*

The solvents used were of the highest purity commercially available and were not purified before use. The same lot of each solvent was used throughout the study. Investigators should be careful of possible differences in solvent toxicity due to either different manufacturers or different production lots. All test compounds were added to the serum at the beginning of the incubation period. Ethyl alcohol (U.S. Industrial Chem. Co.), DMSO (Allied Chem. Co.), acetone (Burdick & Jackson Lab.), Tween 80 (Fisher Chem. Co.) and distilled water were miscible with serum and therefore required only gentle mixing to achieve a homogeneous solution. The water insoluble compounds, corn oil (Atlantic & Pacific Tea Co.) and 10% acetone/90% corn oil, were suspended in serum after sonication for 3 min in an Ultrasonics sonicator (350 W) equipped with a microtip. To remain in suspension for a 2-day period, the corn oil/serum preparations required resonification 24 h after the beginning of culture. The embryos were removed from the corn oil/serum preparations and placed in Trowell's T8 media during the 3-min resonification. The solvent concentrations used ranged from 0.1% to 10%.

Quantitative data are presented here as mean ± S.E.M. for 5 or more independent observations. Statistical significance was determined by analysis of variance procedures comparing treatment groups to controls run on the same day. Where significant effects were indicated $P$-values were determined by using the student's $t$-test [24]. Enumerative data were statistically evaluated using a Fishers exact test [25] and reported as the per cent occurrence.

## RESULTS

The effects of solvents in concentrations ranging from 0.1% to 10.0% on yolk sac growth are summarized in Fig. 1. Final yolk sac DNA, protein and change in yolk sac diameter, were significantly decreased at the 0.5% concentration of Tween 80. Yolk sac DNA, protein and diameter were greatly reduced at the 2.5% level in all groups except corn oil. The increase in yolk sac diameter of the 0.5% acetone and 0.5% acetone/corn oil groups, were significantly reduced even though yolk sac protein and DNA were not affected. Exposure to 0.5% ethyl alcohol decreased yolk sac DNA content but

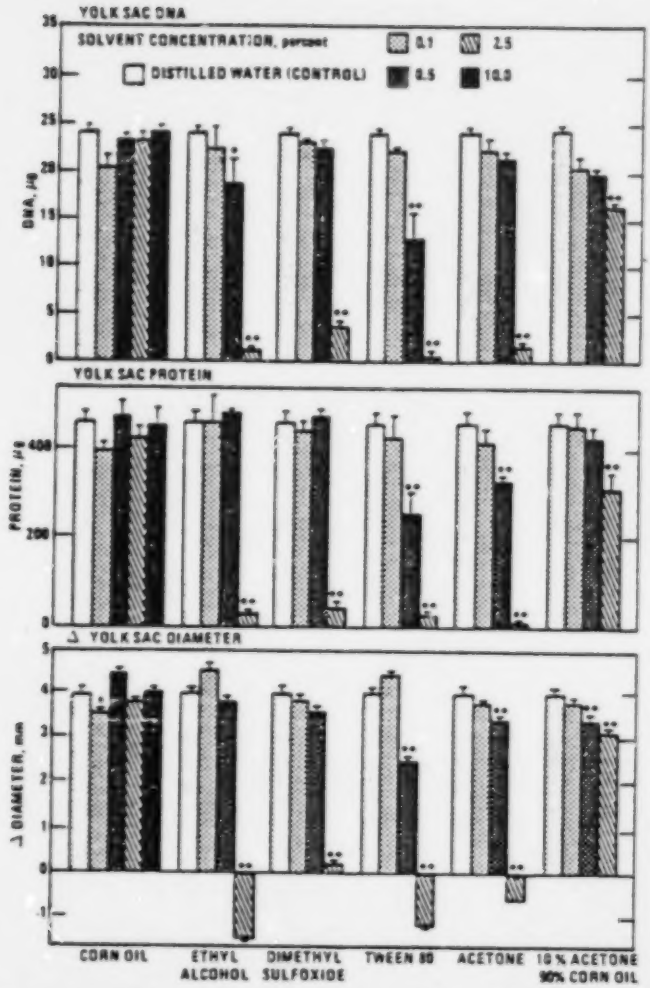


Fig. 1. The effects of 6 solvents on the development of the rat yolk sacs in vitro. Embryos were removed from their dams on day 10.5 and cultured for 48 h in 0.1%; 0.5%; 2.5% or 10.0% of the indicated solvent. Distilled water (10%) was used for the controls. The final yolk sac DNA and protein content and the increase in yolk sac diameter which occurred during culture are shown. Data are presented as mean ± S.E.M. with * = $P \leq 0.01$ and ** = $P \leq 0.001$. The number of samples per group is the same as in Table I.

not the increase in yolk sac diameter or protein content. Although significant ($P < 0.001$) the 2.5% acetone/corn oil treatment group was less toxic than the other water soluble solvents (ethyl alcohol, DMSO, Tween 80 and acetone). Corn oil was remarkably non-toxic to the developing rat yolk sac in vitro.

Growth of cultured embryos exhibited a very similar pattern of solvent

TABLE I

THE EFFECTS OF VARIOUS SOLVENTS ON POST-IMPLANTATION RAT EMBRYOS IN-VITRO

The mean ± S.E.M. or the percent occurrence is given. The number of embryos evaluated for each of the 4 parameters is shown in parentheses. Dead embryos were excluded from the normal/abnormal classification. * = $P < 0.05$, ** = $P < 0.01$, ** = $P < 0.001$, *NM* = not measurable.

| Solvent | Dosage (% culture media) | Crown-rump length (mm) | Head length (mm) | % abnormal embryos | % dead embryos |
|---|---|---|---|---|---|
| Distilled water | 10.0 | 5.35 ± 0.14 (32) | 2.80 ± 0.07 | 12.5 | 0.0 |
| Corn oil | 0.1 | 5.49 ± 0.27 (9) | 2.84 ± 0.17 | 22.2 | 0.0 |
| | 0.5 | 5.56 ± 0.13 (6) | 2.83 ± 0.03 | 16.7 | 0.0 |
| | 2.5 | 5.40 ± 0.18 (13) | 2.78 ± 0.09 | 25.0 | 7.7 |
| | 10.0 | 5.37 ± 0.03 (6) | 2.83 ± 0.03 | 40.0 | 16.7 |
| Ethyl alcohol | 0.1 | 5.15 ± 0.28 (6) | 2.53 ± 0.20 | 33.3 | 0.0 |
| | 0.5 | 4.68 ± 0.39 (7) | 2.36 ± 0.20 | 71.4** | 0.0 |
| | 2.5 | NM (5) | NM | NM | 100.0*** |
| Dimethyl sulfoxide | 0.1 | 5.80 ± 0.26 (6) | 2.95 ± 0.13 | 16.7 | 0.0 |
| | 0.5 | 5.22 ± 0.12 (6) | 2.65 ± 0.02 | 16.7 | 0.0 |
| | 2.5 | 1.93 ± 0.12***(10) | 0.79 ± 0.10*** | 100.0 | 90.0*** |
| Acetone | 0.1 | 5.45 ± 0.34 (10) | 2.76 ± 0.22 | 22.2 | 0.0 |
| | 0.5 | 4.98 ± 0.15 (10) | 2.70 ± 0.17 | 50.0* | 0.0 |
| | 2.5 | NM (6) | NM | NM | 100.0*** |
| Tween 80 | 0.1 | 5.16 ± 0.48 (5) | 2.67 ± 0.33 | 0.0 | 0.0 |
| | 0.5 | 4.03 ± 0.54*** (5) | 1.98 ± 0.34*** | 60.0* | 0.0 |
| | 2.5 | 2.20 ± 0.15*** (5) | 1.00 ± 0.00*** | NM | 100.0*** |
| 10.0% Acetone: 90.0% Corn oil | 0.1 | 5.72 ± 0.40 (6) | 2.97 ± 0.27 | 0.0 | 0.0 |
| | 0.5 | 5.53 ± 0.37 (6) | 2.90 ± 0.03 | 33.3 | 0.0 |
| | 2.5 | 4.48 ± 0.21 (5) | 2.40 ± 0.10 | 80.0** | 0.0 |

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, NM = not measurable.

toxicity (Fig. 2). Significant decreases were seen at the 2.5% level in all groups except corn oil. Embryo protein and DNA were greatly reduced following exposure to 0.5% of Tween 80. However, only embryo DNA values were significantly reduced by exposure to 0.5% ethyl alcohol.

Embryonic growth was further evaluated by measuring crown-rump length and head length (Table I). The corn oil group was not different from controls at any of the 4 dose levels. A dose-related response was evident after embryos were cultured with the 5 other solvents. Because of the 100% embryolethality and tissue deterioration crown-rump and head length could not be measured in the embryos grown in serum containing 2.5% ethyl

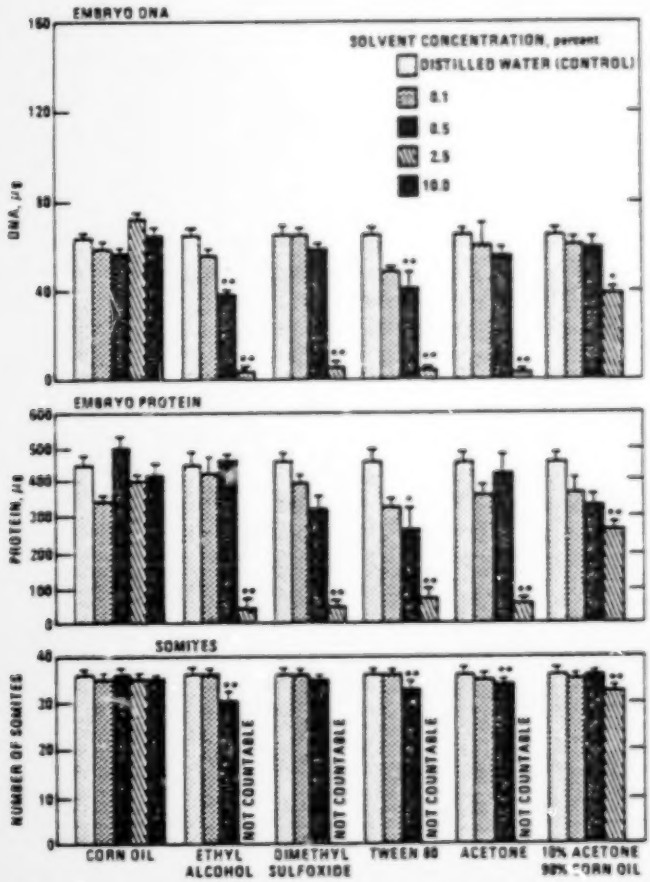


Fig. 2. Growth and differentiation of rat embryos in vitro. Day 10.5 embryos were cultured for 2 days in rat serum containing solvents at concentrations of 0.1%; 0.5%; 2.5% or 10.0%. Ten per cent distilled water was used for controls. The final embryonic DNA and protein content and somite number are illustrated. Data are presented as mean ± S.E.M. with * = $P \leq 0.01$ and ** = $P \leq 0.001$. The number of samples per group is the same as in Table I.

alcohol and acetone. Embryo culture in 2.5% acetone/corn oil and DMSO decreased embryo crown-rump and head length. At a concentration of 0.5%, acetone had no statistically significant effects while ethyl alcohol and Tween 80 decreased both crown-rump and head length.

The number of somites present after 48 h of culture of day 10.5 rat embryo explants was used as a measure of embryonic growth and differentiation (Fig. 2). Embryos treated with 0.5% ethyl alcohol, Tween 80 and acetone had decreased somite counts as compared to distilled water controls. With the exception of corn oil and acetone/corn oil, at a concentration of 2.5% all solvents caused such a marked retardation of embryonic differentiation and deterioration of embryonic tissue that somites could not be counted.

The solvent-induced incidence of morphological abnormalities and mortality are presented in Table I. At 2.5% of the serum incubation media ethyl alcohol, DMSO, acetone and Tween 80 caused 90–100% embryonic death. At a concentration of 0.5%, no embryo lethality occurred in any of the solvent treatment groups. At this dose, acetone, Tween 80 and ethyl alcohol caused significant incidences of structurally abnormal embryos (50.0, 60.0, 71.4%, respectively, as compared to 12.5% for controls). Corn oil and acetone/corn oil were without effects at a concentration of 0.5%. Acetone/corn oil caused significant dysmorphogenesis at 2.5% (80% of the embryos), while 2.5% corn oil did not cause statistically significant dysmorphogenesis (25% abnormal).

Dysmorphogenesis of the brain, heart and mandibular arches were the most frequent anomalies seen. Abnormal development of the brain was observed in embryos treated with 0.1% acetone, 0.5% Tween 80, 0.1% and 0.5% ethyl alcohol and 0.5% of the acetone/corn oil mixture. Exposure to DMSO (0.1 and 0.5%), ethyl alcohol (0.1 and 0.5%) acetone (0.5%) and acetone/corn oil (0.5%) caused enlarged pericardia. The mandibular arches of embryos treated with 0.1% acetone, 0.5% ethyl alcohol, 0.1% corn oil and 2.5% acetone/corn oil appeared edematous on gross examination. Representative micrographs of live specimens of day 10.5 rat embryos cultured for 48 h in 10.0% distilled water, or 0.5% Tween 80, DMSO, ethyl alcohol, acetone/corn oil and corn oil are presented in Fig. 3a–f. The incidence of embryonic anomalies in groups treated with 0.5% ethyl alcohol, DMSO, acetone, Tween 80, and acetone/corn oil was as follows: swollen (edematous) mandible < enlarged pericardium < retarded brain development and small for date embryos. The incidence of swollen mandibles and enlarged or edematous pericardia was approximately the same as in the control groups. Retarded development of the telencephalon and small embryos (as determined by gross observation) are the major anomalies seen in treatment groups having elevated numbers of abnormal embryos. In most cases small embryos did not have retarded telencephalons. Telencephalic development was normal in 72% of the embryos judged to be small.

The morphology of embryos cultured in 0.5, 2.5 and 10.0% corn oil is illustrated in Fig. 4. Swollen mandibles, small embryos and, at the higher doses, retarded telencephalic development were characteristic of corn oil dysmorphogenesis. Mandibular arches appear edematous as compared to controls. Small for date embryos seem otherwise normal.

Fig. 3. Effects of 0.5% (v./v.) of 5 different solvents on rat embryo morphogenesis in vitro: (a) distilled water control; (b) Tween 80 caused abnormal development of the brain (arrow); (c) dimethyl sulfoxide; (d) ethyl alcohol treated embryos exhibited abnormal brain development (top left arrow) with possible unilateral hydrocephaly (top right arrow); (e) 10% acetone/90% corn oil caused pericardial edema (arrow); and (f) corn oil. Magnification ×15.

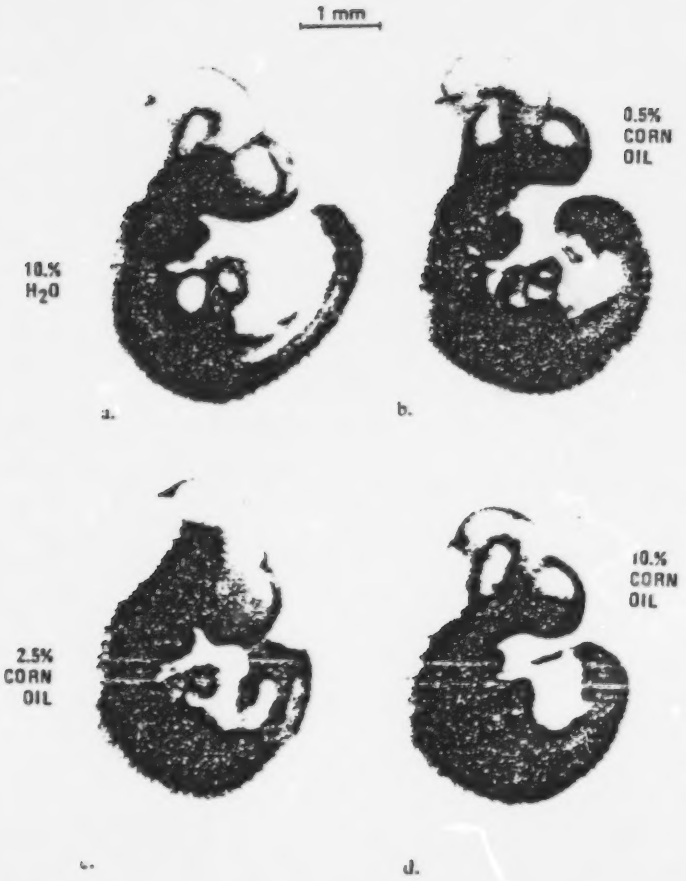


Fig. 4. Effects of 3 different corn oil concentrations on the differentiating rat embryo in vitro: (a) 10% distilled water (control); (b) 0.5% (v/v); (c) 2.5% (v/v); (d) 10.0% (v/v). Magnification × 12.5. Overall morphogenesis is good.

## DISCUSSION

Corn oil in serum was a non-toxic solvent. However, corn oil did increase the incidence of abnormal embryos at 2.5 and 10.0% (Table I). To make an in vivo comparison, 10% (v/v) corn oil is roughly equivalent to a dose of 23 ml of corn oil/250 g rat (v/w). Resonification is required to maintain the suspension of corn oil in culture serum for the full 48-h culture period. Corn oil is far less toxic and dysmorphogenic than all other solvents tested.

Suspensions of 10% acetone/90% corn oil were the second least toxic solvent and had little dysmorphogenic effect at 0.5% concentration. The in vitro embryotoxicity and dysmorphogenic effect of various doses of acetone/corn oil mimics the effect of the acetone content of the mixture. Thus 2.5% acetone/corn oil (0.25% acetone) is somewhat less toxic and dysmorphogenic than pure 0.5% acetone.

All water insoluble chemicals do not dissolve directly in corn oil. However, solutions in acetone (or other volatile organic solvents like benzene) may be prepared, diluted with corn oil, $N_2$ gently bubbled through to evaporate the volatile organic solvent, leaving the solute dissolved in corn oil. Traces of organic solvents like acetone left in corn oil should not greatly affect normal embryonic growth and differentiation.

Of the water miscible solvents, DMSO was less toxic and dysmorphogenetic than either ethyl alcohol or acetone and all 3 were clearly less deleterious than Tween 80. DMSO is known to be teratogenic and embryolethal in hamsters [26] and mice [12], but in the rat and rabbit embryolethality occurred without a teratogenic effect [12]. DMSO caused no discernible effects at 0.5% but was greatly toxic at concentrations of either 1.0% (Kitchin and Ebron, unpublished data) or 2.5% in the serum incubation media. Thus using DMSO in the range of 0.25–0.5% has little margin of safety (<4.0). For example, in studies where a toxic solute is dissolved in 0.25–1.0% DMSO (toxic solvent) the overall effects cannot be certainly attributed to the toxic solute alone. Even if DMSO per se has no effects, that would not rule out possible addition or potentiation interactions between DMSO solvent toxicity and solute toxicity. At a solvent concentration of 0.5% only 25 $\mu$l of solvent can be added to 5000 $\mu$l of serum media. To add all water insoluble chemicals to in vitro embryo culture in the desired concentrations this 25-$\mu$l volume may not be sufficient.

Brown et al. [27] have cultured 9.5-day rat embryos in 0.15 and 0.30% ethyl alcohol for 2 days. Their study showed no effect at 0.15% but a significant decrease in embryo growth and morphological score was noted at 0.30% ethyl alcohol. Therefore, our observed dose response relationship with ethyl alcohol and that of Brown et al. [27] are compatible. Some in vivo studies in mice have shown ethyl alcohol to be teratogenic [13] while others [28] have found no effects other than retarded fetal growth [29].

In our embryo culture dose response study ethyl alcohol, acetone, Tween 80 and acetone/corn oil caused dysmorphogenesis at doses below embryolethality. DMSO in the serum incubation media induced embryonic dysmorphogenesis and embryolethality at 2.5% but not at 0.5%.

In conclusion, dose response studies of solvent toxicity in rat in vitro embryo culture have been performed. The toxic and dysmorphogenic data of this in vitro embryo culture study permit the ranking of the 6 solvents in order of their increasing deleterious effect: corn oil < acetone/corn oil < DMSO < ethyl alcohol, acetone < Tween 80. The maximal solubility of various test agents in the 6 solvents studied here may vary greatly. Therefore investigators may choose to use a more toxic solvent if the test agent

solubility in that solvent is particularly favorable. Using these 6 tested solvents of known rat embryo toxicity and dysmorphogenesis, future whole embryo culture can be performed with water insoluble chemicals. Any of the 4 water miscible solvents (at 0.1%) or a sonicated suspension of corn oil in serum (up to 2.5%) met the criteria of a non-toxic and teratogenic water insoluble compound delivery system for in vitro embryo culture. Preliminary studies are already under way in this laboratory to develop a fully integrated whole rodent embryo culture system which contains both a water insoluble chemical delivery system and an adult hepatic activation system.

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